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(57) Abstract

Disclosed are formulations for use in preparing efficient and stable liposomes for therapeutic use according to an extrusion process. These formulations are effective in increasing the capture efficiency and stability of the liposomes with respect to water soluble therapeutic nucleotides, such as AZT monophosphate. Effective formulation characteristics include the presence of unsaturated acyl chains in phospholipids and effective amounts of negatively charged phospholipids as well as sterols in the lipid phase, the use of hydration buffers having an approximately neutral or acidic pH, and a favorable formulation concentration of therapeutic agent. Disclosed also are processes for preparing liposome preparations by means of freeze-thaw and extrusion cycles, followed by filtration. Liposomes prepared by the extrusion process according to optimum formulations show high capture efficiency for nucleotides as well as extended stability with respect to leakage of nucleotides when exposed to serum.

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IMPROVED LIPOSOMAL FORMULATIONS OF NUCLEOTIDES AND NUCLEOTIDE ANALOGUES

Field of the Invention

The invention relates to formulations for liposomal drug delivery systems and specifically to formulations for liposomal delivery of therapeutic nucleotide analogues.

Background of the Invention

Liposomal drug delivery systems increase the potency of therapeutic agents they contain by focusing their activity to selected biological sites. The use of liposome systems also makes it possible to administer in this way agents that are too toxic or perhaps too expensive for conventional parenteral introduction.

Infectious diseases usually infect only a specific small subset of all the different cell types available in the host organism. For instance, herpes virus prefers cells of the central or peripheral nervous system, leishmaniasis is an infection of the macrophage, and malaria infects red blood cells.

The virus infection caused by human immunodeficiency virus (HIV), known as AIDS, is a more complex example because it is capable of infecting both macrophages and lymphocytes. An infection of the lymphocyte kills the cell, and this loss of critical lymphocyte populations leads to a compromised immune system, making the patient susceptible to opportunistic infections and to cancer. Infected macrophages, on the other hand, remain viable, and the macrophage population in HIV infected patients persists as a productive reservoir of infectious HIV virus capable of releasing infectious virus into the circulation of the AIDS patient where it can infect and kill more lymphocytes. For this reason it is desirable to develop a therapy which preferentially treats the macrophage reservoir of the HIV virus.

Liposomes are ideal vehicles for delivering antimicrobial agents specifically to macrophages, because after injection they are spontaneously cleared by the macrophage component of the reticuloendothelial system. Presumably, any antiviral agent stably incorporated into liposomes would be preferentially delivered to the macrophages, and an infection residing in the macrophage pool would be expected to be preferentially treated.

The clinical effectiveness of liposome preparations depends in large part on the dose of therapeutic agent which they can deliver to a sensitive physiological site. The delivered dose is determined both by the concentration of drug encapsulated within the liposomes during their manufacture, as well as the ability of the liposomes to retain the drug during storage. It is also determined by the ability of the liposomes to remain stable and to retain the drug long enough in the interstitial fluid and in serum.

The amount of drug encapsulated within the liposomes in the course of any specific manufacturing procedure depends on a number of characteristics of the liposome formulation, as well as the size of the liposomes.

Liposomes as originally described by Bangham, A.D. et al., J. Mol. Biol. 13:238-245 (1965) are formed when a lipid film, comprising polar lipid species such as phospholipids, are hydrated by a volume of aqueous fluid. The phospholipids align themselves to form a bilayered membrane, their hydrophilic heads facing outwardly in association with water molecules on each side and their hydrophobic fatty acid tails facing the interior of the membrane in association with each other. Hydrophobic forces also urge phospholipid bilayers to further associate, forming spheres, or lipid vesicles, which are the structural units of liposomes. In the process of formation the vesicles entrap a volume of the surrounding aqueous fluid, thereby incorporating any dissolved solute. The bilayer of the liposomes, like the membrane of cells, is quite permeable to water, but relatively impermeable to

many water soluble solutes, so entrapped agents can theoretically remain so until biological delivery. The amphipathic behavior of phospholipids and permeability characteristics of the phospholipid bilayer thereby provide a mechanism by which therapeutic agents, such as drugs, can be loaded into liposomes.

The primary liposomes formed on hydration of a lipid film may be of various sizes, but usually have diameters of at least 1.0 micron or more. They may be multilamellar, having several concentric bilayer vesicles, or unilamellar.

The aqueous contents of liposomes can be delivered into the cell cytoplasm by two mechanisms. Liposomes containing the entrapped substance can be engulfed by the cell as a whole entity, by a phagocytic or endocytic process. Alternatively, the liposome can fuse with the cell surface and inject its aqueous contents past the plasma membrane. Accordingly, lipid soluble agents, incorporated into the lipid film prior to hydration, may also be delivered by liposomes. Multilamellar vesicles may fuse with membranes or disintegrate in vivo one layer at a time and for that reason are useful as slow release vehicles. Unilamellar liposomes are more efficient carriers of water soluble agents primarily because of their proportionately greater fluid cargo. Large unilamellar liposomes, by reason of their geometry, carry more fluid and hence more solute per unit weight of lipid.

Optimization of the loading capacity of liposomes for water soluble agents consists in part of efforts to convert both multilamellar and small unilamellar liposomes formed in the lipid hydration process to unilamellar vesicles of the largest effective diameter. It is known that liposomes having diameters less than about 4 microns and optimally between 0.1 and 0.3 microns are most desirable for parenteral use. Vesicles larger than 4 microns present the untoward prospect of causing an embolism in small capillaries. Vesicles 0.2 microns in diameter or less are particularly attractive because they can be sterilized with

terminal filtration as the last step in the manufacturing process. These smaller vesicles may also be taken up more efficiently by phagocytic cells. A liposome preparation can be sized to a narrow distribution of diameters within a biologically effective range by freeze-thaw treatment combined with repeated extrusion, under pressure, through porous filters. Freeze-thaw cycles fuse small unilamellar liposomes into larger ones and also convert multilamellar to unilamellar liposomes. Filtration also converts multilamellar liposomes, and at the same time reduces the diameter of those unilamellar vesicles which are substantially larger than the filter pore size. Mayer et al., Biochim. Biophys. Acta 817:193-196 (1985), have been successful in substantially increasing both the trapped volume and trapping efficiency of liposomes by repetitive freeze-thaw cycles alone, and in a related study, Biochim. Biophys. Acta 858:161-168 (1986), describe an optimum protocol further comprising filter extrusion which produces unilamellar systems having high trapping efficiencies and homogeneous size distributions within the biological range.

Loading capacity is affected not only by the size and structure of the liposomes but also by the nature of the solute in the hydration buffer. Studies with Mn^{++} and labeled inulin indicate a nonequilibrium distribution of solute molecules across the lipid bilayer, tending unfortunately to a lower solute concentration within the liposomes. Solute disequilibrium in primary liposomes can be improved by freeze-thawing, but the improvement may be due in part to a reduction in the number of multilamellar vesicles. (Mayer et al., Biochim. Biophys. Acta 817:193-196 (1985)).

Solute trapping efficiency then appears to depend on other factors in addition to physical features such as the favorable size and structure of liposomes. (Szoka, F. and D. Papahadjopoulos, Proc. Natl. Acad. Sci. (US) 75:4194-4198 (1978)). Critical factors affecting transmembrane solute equilibrium must obviously be optimized for each

class of solutes.

Nucleotide analogues are highly potent anti-proliferative agents which can be effectively delivered by liposomes. These agents may kill rapidly dividing cells by becoming incorporated into metabolic processes along with endogenous natural nucleotides and subsequently blocking a biochemical pathway because of their structural incapacity to participate in a critical pathway reaction. This incapacity is usually the result of a chemical alteration at reactive sites on the molecule. Some nucleoside analogues are specific enough that they only block the replication of the pathogenic microorganism and leave the normal cellular metabolism of the infected cell relatively untouched; in this way these nucleoside can specifically interrupt virus replication without killing the infected cell or without substantially interrupting normal cellular metabolism.

Nucleotides are involved in a variety of biochemical activities, for example, as energy transferring ATP, dinucleotide enzyme cofactors, and as elements of polynucleotides such as tRNA, and the encoded DNA and RNA. Nucleotide analogues destroy the cells they enter by replacing the naturally occurring nucleotides to disrupt these activities.

Anti-neoplastic and anti-microbial nucleotide analogues are most conveniently administered and fed into these pathways as nucleotide monophosphates (N-MP's), comparatively acidic (pK_1 of NMP's = 1.0) molecules having a high negative charge density. Nucleoside di- and tri-phosphates can also be used, as well as higher order polyphosphates. Optimal liposomal loading and storage of these molecules is unlikely to be predictable from studies using neutral molecules or small charged species.

It is an object of the invention to develop a practical liposomal pharmaceutical product containing encapsulated phosphorylated nucleoside. The advantages of using phosphorylated nucleoside, as opposed to

unphosphorylated nucleoside, has been previously disclosed in a prior patent application to Hostetler, U.S. Application Ser. No. 216,412, which is hereby incorporated by reference. The formulation development process disclosed herein has uncovered factors about the formulation composition which are prerequisite to the development of a practical pharmaceutical agent for the applications disclosed herein.

It is therefore an object of the invention to identify the optimum characteristics of the liposome formulation, with respect to both the lipid and aqueous phases which determine the capture, in vivo stability, and storage of nucleotide and their analogues within the lipid vesicles of conventional liposome preparations.

Summary of the Invention

The present invention provides formulations for preparing liposomal preparations suitable for the parenteral delivery of nucleotides, preferably therapeutic nucleotide analogues. The characteristics of these formulations are optimized to increase the capture efficiency, or the concentration of these nucleotides which may be loaded into liposomes, as well as the capture stability, or resistance to leakage of the nucleotides from the liposomes during storage and when introduced into the bloodstream. The invention also provides a process for preparing liposomes from these formulations by which the capture efficiency for nucleotides within lipid vesicles is also enhanced. In addition the invention identifies critical in vitro and in vivo stability parameters and discloses methodology for their optimization.

Thus, in accordance with one aspect of the present invention, there is provided a formulation for preparing liposomes, comprising a lipid phase comprising at least one neutral phospholipid species and at least one negatively charged phospholipid species in which the concentration of the negatively charged species with respect to total lipids

is at least about 1 mole percent, and the phospholipids have acyl groups, at least some of which are unsaturated; a hydration buffer having a pH of from about 4.0 to 8.0; and a nucleotide species in the hydration buffer which is to be encapsulated within the liposomes. In an alternate embodiment, there is provided a liposome formulation, similarly comprising a lipid phase comprising neutral and negatively charged phospholipids having acyl groups, at least some of which acyl groups are unsaturated, in which the concentration of the negatively charged phospholipid species with respect to total lipids in the lipid phase is at least about 5 mole percent, the hydration buffer has a pH of from about 4.0 to 6.0, and the hydration buffer contains a nucleotide species to be encapsulated.

In preferred embodiments the lipid phase of the formulation further comprises a sterol, and in a particularly preferred embodiment, the sterol is cholesterol. The hydration buffer preferably contains a nucleotide species at a concentration between about 20 mM and 120 mM, and most preferably about 50 mM. Also in preferred embodiments, the nucleotide species is a nucleotide analogue, most preferably AZT monophosphate.

In one embodiment of the invention, the pH of the hydration buffer is from about 6.0 to 8.0, and most preferably 7.5; in another preferred embodiment, the pH of the hydration buffer is from about 4.0 to 6.0, and most preferably 5.0.

In one particularly preferred embodiment, the formulation comprises a lipid phase comprising at least one neutral phospholipid, at least one negatively charged phospholipid, and cholesterol, wherein the negatively charged phospholipid is from about 2 to 4 mole percent of total lipids in the lipid phase, the cholesterol is from about 25 to 35 mole percent of total lipids in the lipid phase, and the phospholipids have acyl groups, at least some of which are unsaturated; a hydration buffer, having a pH of from about 6.0 to 8.0 and containing a nucleotide

species in the hydration buffer at a concentration of from about 20 mM to 90 mM.

In another particularly preferred embodiment, the formulation comprises a lipid phase comprising at least one neutral phospholipid, at least one negatively charged phospholipid, and cholesterol, wherein the negatively charged phospholipid is about 10 mole percent of the total lipids in the lipid phase, and the phospholipids have acyl groups at least some of which are unsaturated; a hydration buffer having a pH of from about 5.0 to 6.0; and a nucleotide species in the hydration buffer at a concentration of from about 20 mM to 90 mM.

In preferred embodiments, the concentration of lipids in the formulation immediately after hydration of the lipid film is from about 90 mM to 300 mM; the unsaturated acyl groups of the phospholipids are oleyl esters, or comprise both oleoyl and palmitoyl esters; and about 3 to 30 mole percent of the total lipids of the lipid phase are negatively charged phospholipids. The negatively charged phospholipids are preferably phosphatidyl glycerols.

The hydration buffer solution of the formulation is preferably isotonic, having an osmolarity greater than 200 mos. Substances used to increase the osmolarity of the solution must be physiologically acceptable agents, and accordingly, preferred osmotic agents are sodium chloride or a neutral sugar such as, for example, sorbitol.

According to another aspect of the invention there is provided a process for preparing liposomes comprising providing a lipid phase comprising at least one neutral phospholipid and at least one negatively charged phospholipid, wherein the negatively charged phospholipids are at least 1 mole percent of the total lipids in the lipid phase, and the phospholipids have acyl groups, at least some of which are unsaturated; providing a hydration buffer having a pH between about 4.0 and 8.0, and preferably between 6.0 and 8.0, and containing a nucleotide species; contacting said lipid phase with said hydration

buffer, whereby primary liposomes are formed; and adjusting the size of said primary liposomes by extrusion through a filter of uniform pore size. A preferred embodiment of the process further comprises the steps of freezing and then thawing the liposome preparation prior to the extrusion step. In a particularly preferred embodiment, the steps of freezing and thawing followed by extrusion are repeated at least once, and finally the extrusion step is repeated at least once. The pores of the extrusion filter are preferably about 0.200 microns in size.

In any of the foregoing processes, the nucleotide may be a therapeutic nucleotide analogue, such as, for example AZT monophosphate.

The invention further includes liposomes prepared according to any of the processes provided.

According to yet another aspect of the invention, there is provided a liposomal preparation comprising liposomes having a lamellar structure comprising at least one neutral phospholipid species and at least one negatively charged phospholipid species, the negatively charged phospholipid being at least about 1 mole percent of the total lipids in the liposome, the phospholipids having acyl groups, at least some of which are unsaturated; a nucleotide species substantially captured within the liposomes; and a physiologically acceptable buffered aqueous phase having a pH between about 4.0 and 8.0. In an alternate embodiment, the negatively charged phospholipids are at least about 5 mole percent of total lipids, and the hydration buffer has a pH of from about 4.0 to 6.0. In a preferred embodiment, the lamellar phase comprises a sterol, such as for example cholesterol. In a particularly preferred embodiment, the negatively charged phospholipid is a phosphatidyl glycerol, and at least some of the acyl groups of the phospholipids are unsaturated. These acyl groups may preferably be oleoyl esters. In yet another preferred embodiment, the nucleotide species captured within the liposomes is a therapeutic nucleotide analogue, and in a

particularly preferred embodiment, the therapeutic nucleotide analogue is AZT monophosphate.

The invention further provides liposome preparations in which the liposomes are substantially the same size, that is, having diameters with a narrow distribution about the mean diameter. In a particularly preferred embodiment, the mean diameter of the liposomes is approximately 0.200 microns (facilitating sterile filtration, a final manufacturing step). Preferably the liposomes of these preparations are also unilamellar. The liposomes of the invention have substantial stability in serum against leakage of entrapped nucleotide, retaining over 80% of the nucleotide within the liposomes for a period of at least 24 hours when stored together with serum at about 37°C.

In addition to having superior serum stability, the liposome formulations of the present invention have excellent shelf-life, resulting in a projected shelf-life of greater than 2 years at 5°C. Shelf-life stability is judged both by the amount of nucleoside that leaks out of the vesicles upon storage, as well as on the chemical stability of the lipid components.

Detailed Description of the Invention

We have found that the manufacturing, capture and stability of soluble nucleotides and their analogues within the lipid vesicles of liposomes is enhanced by optimizing relevant critical parameters of the liposome formulation.

Relevant parameters of liposome formulations include the nature and structure of lipid phase components together with their total and proportional concentrations. They further include the nature and concentration of components of the buffered hydration solution, together with its pH and osmolarity. Finally, it is known that capture efficiency is affected by the mean diameter of the lipid vesicles.

Conventional liposome-forming lipids are substantially composed of an amphipathic phospholipid species, such as a phosphatidyl choline. The lipid mixture also may include a

negatively charged phospholipid such as, for example, phosphatidyl glycerol as well as an equal or lesser amount of a sterol, such as cholesterol. Liposome-forming phospholipids are commonly isolated from egg yolk, bovine brain or other natural sources. Alternatively they can be chemically synthesized. (Hope, M. et al., Biochim. Biophys. Acta 812:55-65 (1985)). The fatty acid components of naturally occurring phospholipids are both saturated and unsaturated.

The conventional hydration solution is buffered to a neutral pH, usually with phosphates or other common physiological buffer system. The hydration buffer may be brought to isotonicity with NaCl, a polyhydroxyl compound such as a sugar, or any other biocompatible agent.

According to the present invention, liposome formulation parameters have been optimized to maximize both the loading of nucleotide analogues during manufacturing into liposomes and the stability of the liposome preparations during storage. The effects of the variations of formulation parameters measured include: 1) the ratio of entrapped drug to lipid and 2) the concentration of the nucleotide in the final liposome preparation, and 3) the stability of the liposomes and the entrapped drug during storage, as determined by the leakage of drugs from the liposomes, the amount of dephosphorylated nucleotide, and the extent of fatty acid oxidation and fatty ester hydrolysis in the liposome phospholipids.

The formulation development process disclosed herein has uncovered factors about the formulation composition which give rise to less than 20% leakage in 80% serum after 24 hours at 37°C. And furthermore, the formulations have a 2 year shelf-life at 5% of the initial labeled strength (i.e. less than 5% leakage).

The stability of nucleoside incorporation into liposomes after the liposomal preparation has been introduced in vivo can be predicted by in vitro incubation in the presence of high concentrations (about 80%) of

serum. The required stability may depend on the particular therapeutic application. For instance, a 24 hours incubation at 37°C in serum which gives rise to less than 50% leakage, might be regarded for some applications as sufficiently stable; while in others a leakage of less than 25% would be required.

For the final product to be suitable as a practical pharmaceutical agent, the liposome formulation must also retain the therapeutic agent encapsulated during storage. Pharmaceutical products typically have 2 year shelf-life at 95% of the labeled strength. As a general guideline then, the final liposome product should have less than 5% leakage after 2 years under acceptable storage conditions.

Preparation of Liposomes

Primary liposomes were prepared from various formulations according to the conventional procedure as described, for example, by Bangham, A. et al., J. Mol. Biol. 13:238-247 (1965). Suitable phospholipids and phospholipid-related material for the preparation of liposomes include phosphatidyl choline, phosphatidyl ethanolamine, lysolecithin, lysophosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, sphingomyelin, cephalin, cardiolipin, phosphatidic acid, cerebrosides and dicetyl alcohol. In the liposomes of the present invention, phosphatidyl choline is preferred. Synthetic phospholipids were used as well as those purified from natural sources. The fatty acid groups of the synthetic phospholipids, as indicated at the end of Table I, were mono-unsaturated, and had a chain length of either 16 or 18. Alternatively, phospholipids having greater degrees of unsaturation, for example, having from 1 to 6 double bonds and those with chain lengths of from 12 to 24 carbons may be used. Proportions of lipids are expressed as mole percent of total lipids and were preferably about 70 mole percent phosphatidyl choline and 30 mole percent cholesterol. Negatively charged phospholipids may be added

to the lipid phase in concentrations up to about 50 mole percent. In a preferred embodiment, a negatively charged species is phosphatidyl glycerol, which is added to the lipid phase in the amount of at least about 1 mole percent, and preferably at least about 3 mole percent of the total lipids. The primary liposomes are sized to homogeneity, or at least to a narrow distribution of diameters within the biologically suitable range, by a freeze-thaw-extrusion process essentially according to Mayer, L. et al., Biochim. Biophys. Acta 858:161-168 (1986), as described in Example 1. The liposomes may be frozen by chilling to a temperature at which the fatty acid chains are no longer fluid. Suitable freezing conditions may be provided by a bath of dry ice in acetone, an alcohol, or other suitable solvent which will provide a fluid system at the melting point of dry ice, about -70°C. Under these conditions the aqueous hydration buffer also freezes. Thawing of the preparations may be conveniently carried out at room temperature. Alternatively, the preparation may be frozen by immersion in liquid nitrogen and thawing may be carried out in a water bath to provide a temperature above room temperature at, for example, 37°C.

Suitable filters for the extrusion may be of any type prepared for microfiltration, such as those manufactured by Nucleopore, Inc. (Pleasanton, California), having a uniform pore size and manufactured of a non-contaminating material. Filters used for the procedure described in Example 1 were typically of pore sizes either 50 nm or 200 nm. Filters having larger or smaller pore sizes may be used to size preparations of liposomes to larger or smaller mean diameters. Preferably the filters have a uniform pore size in the range of from about 30 to 400 nm. Most preferably, the filter has a pore size of about 200 nm. The liposomes may also be sized by extrusion through a ceramic filter such as, for example, one of the type described in U.S. Patent No. 4,737,323 to Martin. The extrusion is carried out under pressure to facilitate the

flow of the preparation. Suitable pressures may range from 100 to 700 psi. The pressure applied is greater or lesser as required to extrude liposomes through filters of different pore sizes.

The freeze-thaw-extrusion process as described in Example 1 was used to produce the liposome preparation of Table I. The effective process typically consists of from one to three freeze-thaw-extrusion cycles followed by one or two additional filter extrusion passes. The process elements of freeze-thawing and extrusion may be manipulated or extended as required to bring the liposomes of the preparation to within a required range of size distribution.

Size of the individuals liposomes may be determined, as in Example 2, by means of an N4MD submicron particle size measuring device (Coulter, Amherst, Mass.) which operates on the principle of quasielectric laser light scattering. Lipid vesicle size may also be determined by means of equivalent instrumentation, such as, for example, electron microscopy or gel filtration chromatography.

Parameters affecting Capture Efficiency and Drug to Lipid Ratio or Optimal Loading of the Lipid Vesicles

Capture efficiency may be determined in absolute terms as the total amount of solute entrapped within the lipid vesicles of a volume of the liposome preparation, or in relative terms as the ratio of entrapped nucleotide to liposomal lipid. At high lipid concentrations, that is, above 300 mM, capture efficiency in the absolute sense approaches 100%, because all of the solvent volume is enclosed in lipid; however capture efficiency in the relative sense may decrease under the same conditions.

A manufacturing procedure with high capturing efficiency in the absolute sense is generally desirable because it leads to a greater percentage of the raw materials appearing in the final product which translates into a reduced manufacturing cost.

For an effective therapeutic agent, it may be important to achieve a high drug to lipid ratio; this is because for each liposome effectively delivered to its target site, more drug will be delivered when the drug to lipid ratio is higher. In comparing effective formulations (Table I) at a fixed lipid concentration, capturing efficiency and drug to lipid ratio for liposome formulations of nucleotides depends on a number of formulation parameters which appear to be interdependent.

The trapping efficiency of liposome preparations is due in part to the nature of the solute in the hydration buffer, at constant lipid concentration. Figure 1 shows similar capture efficiency of liposomes for the antiretroviral agent 3'-azido,2',3'-dideoxythymidine monophosphate (AZT-MP) as compared to adenosine monophosphate (AMP), cytosine monophosphate (CMP), guanosine monophosphate (GMP) and uridine monophosphate (UMP) in identical liposome formulations. Comparative data for the liposomal entrapment of these closely related nucleotides when neutral and acid hydration buffers are used indicates that this effect is independent of pH, the otherwise most sensitive formulation parameter.

Titration curves for AMP and AZT-MP (Figure 2) indicate the effect of substitution is to shift the pK of the first ionizable hydrogen of the phosphate group from 4.2 to 3.0, a more acidic point, and the pK of the second ionizable hydrogen from 6.7 to 7.3, a more basic position. The pH dependent changes in capture efficiency shown in Figures 1, 3,4, 5, and 6 may result from changes in the protonation state of AZT-MP as a function of pH.

Effect of Nucleotide Concentration

The solute entrapping capacity of liposomes appears to be inversely related to the concentration of solute in the hydration buffer during primary liposome formation. Figure 3 shows that under conditions of constant lipid concentration during extrusion, the percent of AZT-MP

captured is inversely related to its concentration in the hydration buffer. For example, in formulations using equal volume of hydration buffer and equal concentrations of total lipid, over 40% of AZT-MP present in 20 mM hydration buffer is incorporated within the liposome, compared to only about 30% of AZT-MP in 90 mM hydration buffer. Thus, increasing the nucleotide concentration in the hydration buffer has a negative impact on the manufacturing process by resulting in less drug captured in the final product.

Figure 4 shows that increasing the nucleotide concentration in the hydration buffer leads to a net advantage with respect to the drug to lipid ratio (top panel), although not in terms of capture (lower panel), in the 0.200 μm vesicles produced by extrusion. Use of high concentrations of nucleotide is limited, however, by the fact that solutions above 140 mM are hypertonic, a physiological contraindication. Capturing efficiency may also be improved in other ways. Accordingly, although AZT-MP may be present in hydration buffers at concentrations ranging from 20 mM to 140 mM, a preferred range of concentration is from 20 mM to 90 mM, and a most preferred concentration is 50 mM. Because of the interrelationship of nucleotide concentration and capture, the concentration of the solute nucleotide in the hydration buffer is held constant when determining optimum pH of the hydration buffer.

Theoretical calculations based on the measured vesicle diameter show that the encapsulation of AZT-MP is in all cases lower than predicted (Figure 4). The deviation from theoretical entrapment increases as the concentration of AZT-MP in the hydration buffer increases. Increasing the phosphatidyl glycerol (PG) content to 10 mole percent increases the capturing efficiency but the deviation from theoretical entrapment still exists. The differences between the best and the worst formulations can be more than 2-fold.

Although the aqueous solubility of AZT-MP is greater than 0.4 M, there may be a quasi-crystalline organization of the soluble AZT-MP due to the tendency for nucleotide molecules to stack and to form base pairs by hydrogen bonding. Optimum loading of this quasi-crystalline, though soluble, AZT-MP may be difficult to obtain inside the confined interior of the phospholipid vesicles, thus resulting in lower capturing efficiency. This phenomenon may be responsible for the deviation from theoretical entrapment that increases as the AZT-MP concentration increases.

In summary, these results indicate that increasing the nucleotide content in the hydration buffer is a factor that can lead to an advantage in terms of the drug/lipid ratio, but a disadvantage with respect to manufacturing.

Effect of Hydration Buffer Composition

The effect of hydration buffer composition on liposome capture may be followed by preparing various liposome formulations (Table II) the conventional freeze-thaw-extrusion process described above and determining the efficiency of entrapping according to the methods described in Example 4: Sections A through D. Results for various liposome formulations are indicated in Table I.

Preferred buffer systems were determined in formulations A through L for AZT-MP incorporation into liposomes at constant AZT-MP hydration buffer concentration and constant lipid composition and concentration. Enhanced entrapment in 0.200 micron vesicles, formulations A through H, occurred according to these studies when the hydration solution was buffered at an acidic pH of 5.0. Supplementary studies showed substantially the same effect when either acetate or succinate was used as the buffering agent (Figures 1, 4, and 5). A comparison of formulations M through P, prepared from high (90 mM) concentrations of nucleotide solute, and Q through T, from low (25 mM) concentrations indicates that the enhanced loading effect

of acidic hydration operates in a comparable way regardless of the concentrate of solute nucleotide. The effect was verified by measurements on a variety of other formulations (Figure 6). The pH of the hydration buffer used in the process of preparing primary liposomes having maximum nucleoside incorporation according to the present invention therefore is in the range of about 5 to 7.4, preferably about 6 to 7, and most preferably the pH is about 6.5.

The hydration buffering agents are physiologically acceptable systems such as, for example, acetate or succinate. The hydration buffer must also be isotonic. Osmolarity of the hydration buffer is preferably greater than 200 mos and most preferably about 300 mos.

Osmolarity may be adjusted by the use of physiologically acceptable agents such as, for example, sodium chloride or a neutral sugar, such as sorbitol. Buffer systems in which the osmotic agent was sodium chloride (NaCl) as well as those containing sorbitol, were equally effective with respect to capturing efficiency and stability of the liposomes.

Effect of Liposome Size

The effect of hydration buffer pH on capturing efficiency appears to be independent of the size of the lipid vesicles. When liposomes were sized through 0.050 micron membranes, formulations I through L, the resulting drug/lipid ratios obtained were less than the corresponding 0.2 micron extruded vesicles (A-H). Comparing drug/lipid ratios for the 0.05 micron vesicles at pH 7.4 and 5.0 shows that pH effect on capturing efficiency still obtains.

Effect of Lipid Composition

The favorable effect of negatively charged lipid species in the lipid bilayer on capture efficiency is apparent from the data of Figures 4 and 7. Phosphatidyl glycerol in lipid vesicles of comparable size prepared from otherwise identical formulations dramatically enhances

capturing efficiency (Figures 4 and 7). Further, a comparison of formulations U,V with W,X indicates that the capture enhancing effect of acidic systems appears not to operate in the absence of negatively charged vesicle phospholipids.

Figure 7 shows that increasing the mole percent of the negatively charged lipid, phosphatidylglycerol (PG), in the vesicles made by extrusion results in a dramatic improvement on the AZT-MP capturing efficiency. The percent encapsulation (top figure), as well as the corresponding drug/lipid ratio (bottom figure), increases as the mole percent of phosphatidylglycerol increases. The difference in capturing efficiency can be more than two-fold. This result suggests that vesicles with a more highly negatively charged surface have a larger internal capture volume.

This effect may result from charge repulsion between the inner surfaces of the vesicle. Neutral vesicles may be relatively collapsed resulting in a lower than predicted capture volume. The addition of charge to the vesicles could cause the inner surfaces to repel each other resulting in a more spherical structure with a larger capture volume. Consistent with this interpretation, it is known that charged lipid bilayer surfaces exhibit a stronger repulsive force than similar neutral surfaces. Shorter range repulsive forces among the individual lipid molecules could also contribute to a more spherical lipid vesicle with a corresponding larger capture volume. Alternatively, these short range repulsive forces could result in an average increase in the square nanometer surface area per phospholipid molecule. This increase would result in a greater number of lipid vesicles per mole of phospholipid and a resulting improvement in the capturing efficiency for vesicles having a higher phosphatidyl glycerol content.

It is postulated that both an acidic environment and charges on the inwardly facing phospholipid phosphate

groups of phosphatidyl glycerol provide charge modulating influences which act to overcome intermolecular repulsion for nucleotides and to increase repulsive forces between nucleotide molecules and lipid bilayer components. These effects act not only to enhance uptake but also to retard leakage from the liposome.

Factors affecting Stability of Liposomal Preparations

Liposome formulations that promote an increased capture efficiency for nucleotide such as optimal buffer composition and lipid composition, as determined above, appear to correlate well with the stability of the lipid vesicles as determined by leakage of the entrapped nucleotide during storage. Leakage was followed during storage of the liposomal preparation by HPLC normal phase gel filtration chromatography as described in Example 3, Section B, to determine quantitatively the amount of extra-liposomal nucleotide which had accumulated in the preparation after storage under different conditions. Using a corresponding assay for total nucleotide (Example 3, Section A), percent leakage can be calculated. The result, as seen in Figures 8 and 9, indicate that at conventional low temperature storage conditions of 5°C, there is virtually no leakage from the liposomes prepared from any of the formulations A through X over a period of 6 months. Under the stress conditions of 15°C temperature, the lipid bilayer membranes appear to become more permeable and the extent of leakage is dependant on the formulation compositions, with the acidic preparations appearing to be more resistant. Measurements of vesicle diameters 2 and 4 weeks after preparation for formulations A through L indicated no significant change.

Stability of the lipid vesicles is promoted by the presence of fluid, unsaturated fatty acids as well as negatively charged species in the phospholipid composition of the structural bilayer. Extrusion vesicles comprised of saturated neutral phospholipids settle out on storage at

all temperatures. Extrusion vesicles comprised of fluid, negatively charged phospholipids, on the contrary, are physically stable and do not settle or aggregate. The lipid vesicles comprised of unsaturated acyl chains are subject to oxidation, as seen in Table III, and the degree of oxidation correlates with leakage from the vesicle. In preferred embodiments, therefore, a liposome preparation comprises lipid vesicles wherein the bilayer membrane comprises a neutral phospholipid such as, for example, phosphatidyl choline, together with cholesterol and a negatively charged phospholipid such as, for example, phosphatidyl glycerol.

Formulation parameters are clearly interdependent, however the data of Table I and related figures 3 to 6 indicate that, under conditions of constant total lipid concentrations in the liposome formulation, higher capture efficiencies for nucleotide liposomal preparations are promoted by the following in order of effectiveness: negatively charged phospholipids in the lipid bilayer; acidic systems; nucleotide concentration in the hydration buffer; and liposome size. The presence of negatively charged phospholipids together with phospholipid having uhsaturated acyl groups appears to promote liposome stability during storage (Table III). Liposome size appears to have an effect also, with liposomes having a diameter of about 200 nm more stable than those having a diameter of about 100 nm.

Factors affecting Serum Stability

The formulations shown in Figures 10 through 12 were prepared to test the effect of cholesterol, PG content, and AZT-MP concentration in the hydration buffer, on the stability of the final products in serum. All studies were carried out on formulations in which the hydration buffer had a pH of 7.4 and in which the liposomes were sized to a mean diameter of about 0.200 μm.

The effect of increasing the concentration of AZT-MP in the hydration buffer resulted in more leakage from the vesicles after addition to serum (Figure 10). This effect results because vesicles comprised of fluid lipids get leaky when exposed to an osmotic gradient. The formulations containing 200 and 400 mM AZT-MP in the hydration buffer were exposed to a significant osmotic gradient after addition to serum.

The majority of the serum mediated leakage observed in these experiments occurred within the first 24 hours after exposure to serum, after which point a plateau was reached and leakage stopped. We speculate that serum components partition into the vesicles to cause leakage, but after equilibrium is reached, no further leakage occurs. The relative integrity of different formulations is evaluated after the 1st 24 hour period.

In all of the experiments shown on the next series of graphs, the control containing free AZT-MP was completely degraded within 24 hours. (The bar for free AZT-MP at 24 hours in this series of graphs is always so small that it is buried in the X-axis).

The data shown on Figure 11 show that the inclusion of cholesterol results in a more rigid bilayer that is less subject to leakage. The second reason results from the high cholesterol content of serum, and from the fact that cholesterol spontaneously transfers from serum components into lipid vesicles. A net flux of cholesterol from serum results in leakage. When cholesterol content of serum is high, there is no net flux into the vesicles, resulting in a low rate of leakage.

Figure 11 shows that increasing the mole percent of PG in the vesicles results in a product that is more sensitive to serum mediated leakage. Thus, increasing PG has a positive impact on the final product by increasing the percent capture and drug/lipid ratio (Figures 4 and 7), but has a negative impact by rendering the vesicles more leaky in serum.

TABLE I

LIPOSOME FORMULATIONS

Formulation	Buffer	Nucleotide Concentration mg/ml	drug/lipid
Lipid: 300 mM POPC/DOPG/Cholesterol (67%/3%/30%); 90 mm AZT-MP			
A 0.200 micron	pH 7.4; Sorbitol (260 mOs)	0.392	0.0658
B	pH 7.4; NaCl (220 mOs)	0.285	0.0629
C	pH 7.4; Sorbitol	0.328	0.0620
D	pH 7.4; NaCl	0.446	0.0640
E	pH 5.0; Sorbitol (240 mOs)	0.848	0.0896
F	pH 5.0; NaCl (230 mOs)	0.734	0.0923
G	pH 5.0; Sorbitol	0.734	0.0921
H	pH 5.0; NaCl	0.744	0.0956
I 0.050 micron	pH 7.4; NaCl	0.308	0.0492
J	pH 7.4; Sorbitol	0.282	0.0478
K	pH 5.0; NaCl	0.633	0.0553
L	pH 5.0; Sorbitol	0.639	0.0619
Lipid: 300 mM POPC/DOPG/Cholesterol(67%/3%/30%); 90 mM AZT-MP			
M 0.200 micron	pH 7.4; Sorbitol	1.010	0.0798
N	pH 7.4; NaCl	1.028	0.0786
O	pH 5.0; NaCl	1.239	0.0918
P	pH 5.0; Sorbitol	1.279	0.0889
Lipid: 300 mM POPC/DOPG/Cholesterol(67%/3%/30%); 25 mM AZT-MP			
Q 0.200 micron	pH 7.4; Sorbitol	0.576	0.0313
R	pH 7.4; NaCl	0.534	0.0280
S	pH 5.0; Sorbitol	0.633	0.0411
T	pH 5.0; NaCl	0.643	0.0353
Lipid: 300 mM POPC/Cholesterol (70%/30%); 25 mM AZT-MP			
U 0.200 micron	pH 7.4; Sorbitol	0.446	0.0379*
V	pH 7.4; NaCl	0.349	0.0244
W	pH 5.0; Sorbitol	0.354	0.0287
X	pH 5.0; NaCl	0.339	0.0275

POPC: palmitoyloleoyl phosphatidyl choline (synthetic)
DOPG: dioleoyl phosphatidyl glycerol (synthetic)
*This is unexpected

TABLE II

COMPOSITION OF COMPONENTS

Extrusion Vesicles:

POPC/DOPG/Cholesterol	67%/3%/30%
POPC/Cholesterol	70%/30%

POPC: palmitoyloleoyl phosphatidyl choline (synthetic)
DOPG: dioleoyl phosphatidyl choline (synthetic)

Buffers:

20 mM Phosphate	0.80% NaCl	pH 7.4	290 mOs
20 mM Phosphate	4.50% Sorbitol	pH 7.4	290 mOs
20 mM Acetate	0.84% NaCl	pH 5.0	290 mOs
20 mM Acetate	4.90% Sorbitol	pH 5.0	290 mOs

TABLE III

STABILITY OF LIPID VESICLES AFTER 3 MONTHS STORAGE at 25°C

Formulation		Oxidation	Nucleotide Leakage
I	pH 7.4	88%	99%
II	pH 7.4	83%	86%
III	pH 5.0	34%	30%
IV	pH 5.0	15%	8%

TABLE IV

FORMULATION COMPOSITIONS FOR SERUM STABILITY

CODE	BUFFER 20mM		pH	AZT-MP μM	LIPID COMPOSITION (mole percent)
AZT-MP Concentrations Study					
18-55-D	Phosphate,	7.40	100*		EPC40/DOPG/Chol (67/3/30)
18-55-E	Phosphate,	7.40	200*		EPC40/DOPG/Chol (67/3/30)
18-55-F	Phosphate,	7.40	400*		EPC40/DOPG/Chol (67/3/30)
Cholesterol Level Study					
18-55-L	Phosphate,	7.40	50		EPC40/DOPG (97/3)
18-55-M	Phosphate,	7.40	50		EPC40/DOPG/Chol (82/3/15)
18-55-N	Phosphate,	7.40	50		EPC40/DOPG/Chol (67/3/30)
DOPG Level Study					
19-61-B	Acetate,	5.0	50		POPC/DOPG/CHOL (67/3/30)
19-61-C	Acetate,	5.0	50		POPC/DOPG/CHOL (60/10/30)
19-61-D	Acetate,	5.0	50		POPC/DOPG/CHOL (40/30/30)

1. All liposomes are 0.200 μM, prepared by the extrusion process.
2. AZT-MP concentration is that in the hydration buffer (μM).
3. Lipid relative concentrations are mole percent; total lipid concentration for all formulations is 200 mM in hydration buffer.
4. NaCl was used as the isotonicifier in all formulations.

* No buffer is present in the drug hydration buffer, since the drug solution itself is isotonic/hypertonic.

EXAMPLE 1

Preparation of Liposomes

Mixtures of phospholipid and cholesterol as indicated in Table I were dissolved in chloroform and evaporated to dryness in a rotary evaporator. Solvent traces were removed by treatment under vacuum for a period of 12 hours. Primary liposomes were then formed by hydrating the lipid film with an aqueous buffer solution, according to Table I, in which a nucleotide analogue was dissolved at concentrations of from 20 mM to 120 mM. Hydration was carried out in a water bath at 60°C, and on a rotating shaker device.

These primary liposomes, containing captured nucleotide analogue, were then sized by a combination of freeze-thaw cycles and extrusion procedures. The freeze-thaw cycles were carried out by immersing the liposome preparation in a bath of dry ice in isopropanol, a temperature of approximately -70°C., until frozen and then allowing the

preparation to thaw in a water bath at 30°C. The primary liposomes were next extruded through a pair of stacked polycarbonate membranes, having a pore size of approximately 0.200 microns, and mount in an extruder device placed in a water bath at 60°C. The extrusions were carried out under pressures of from 300 to 700 psi as required to facilitate flow. The freeze-thaw extrusion cycle was repeated at least once and terminated with multiple extrusion passes.

The mean diameter of the extruded liposomes was determined according to the procedure of Example 2. After the liposomes of the preparation were sized as required, free drug was removed by passing the batch of liposomes over a G-50(fine) Sephadex column (Pharmacia, Piscataway, NJ), and collecting the liposomes in the void volume. To avoid collecting the free drug peak the elution was monitored with a flow cell on a UV detector set at 256 nm.

The pooled void volume was then filtered through a 0.2 micron teflon 66 filter, aliquoted in 0.3 ml fractions into 1 ml serum vials and capped with Teflon stoppers.

EXAMPLE 2

Determination of Liposome Diameter Distribution

Size distribution of individual liquid vesicles and liposome preparation were determined by means of the Coulter N4MD submicron particle sizer (Coulter; Amherst, Mass.). A small quantity of the liposome preparation, usually about 10 μl, was mixed with about 1 ml of phosphate buffered saline having the same pH and concentration as that used in the corresponding liposome preparation. Mixing was carried out carefully to avoid introducing bubbles with the sample which could be read as particles by the particle sizing apparatus. For the runs described herein to determine particle size stability, scattering angle was 90° and refractive index 1.333, viscosity 0.01 poise, and temperature 20°C. Range of determinations: 10 to 1000 nm; and run time was 300 sec.

EXAMPLE 3

Methods to Determine Capture Efficiency and Evaluation of the Stability of a Preparation of AZT-MP Liposomes

A. Total Drug Content

Total drug content, including AZT and AZT-MP, was determined by UV absorbance as follows. A quantity of the aqueous liposome preparation was solubilized with chloroform and methanol, so as to produce a final water/chloroform/methanol ratio of 0.8/2/1. The absorbance of nucleotide in the solubilized sample was read at 265 nm against an appropriate blank.

B. Determination of Free AZT-MP

Free AZT-MP in the liposomal preparation was determined by HPLC gel chromatography under the following conditions:

Column:	TSK 2000SW, 10 $\mu\lambda$, 75 x 300 mm.
Mobile Phase:	5 mM sodium phosphate buffer, pH 6.8 109 mM sodium chloride (290 mOsm)
Flow Rate:	0.50 ml/min, 30 min isocratic
Wavelength:	265 nm
Temperature:	Ambient
Back Pressure:	Approximately 150 psi

A standard curve was constructed by mixing 20 μl of 1 mg/ml AZT-MP standard with 380 μl of the mobile phase and injecting volumes as follows:

5 μl (0.25 μg)
10 μl (0.50 μg)
15 μl (0.75 μg)
20 μl (1.00 μg)

The sample was analyzed by injecting 10 μl of the formulation containing 0.25 to 1.00 μg of AZT-MP. The results of a typical analysis are shown in Figure 1.

Buffer: Sodium phosphate monobasic dihydrate and 7.80 gm sodium chloride 6.36 gm was dissolved in approximately 800 ml of HPLC water. The pH was adjusted to approximately 6.8 with 1N NaOH or 5N NaOH and the volume brought to 1 L with

HPLC water. The prepared buffer was filtered through a 0.45 micron nylon filter.

Column Regeneration: 0.2% acetic acid in 70% methanol was passed through the column at a flow rate of 0.2 ml/minute overnight. The column was stored in 0.2% sodium azide in HPLC water which had been filtered through an 0.45 micron nylon filter.

C. Determination of amount of drug and its chemical integrity within the AZT-MP liposome.

Liposome Sample Preparation: The liposomal formulation was solubilized using methanol to release all the entrapped AZT-MP. An equal volume of mobile phase was added to the solubilized sample prior to injection. Volume of methanol and mobile phase were chosen to adjust the lipid concentration to between 0.5 and 1 mM. For example 10 μl of a formulation comprising 300 mM lipid was dissolved in 240 μl methanol and 250 μl mobile phase added.

Total AZT-MP in the liposome formulation together with any AZT present was determined by HPLC reverse-phase chromatography of the solubilized liposome preparation under the following conditions:

Column:	Vydac C18, 218TP 0.46 x 15 cm. 5 μm
Mobile Phase:	A. 0.1% TFA in HPLC water B. 0.1% TFA in 100% acetonitrile (mix and store in glass only).
Gradient:	0% to 15% B in 15 min; 90% B for 10 min; 0% B for 5 min.
Flow Rate:	1.5 ml/min
Wavelength:	265 nm
Temperature:	Ambient
Back Pressure:	about 600 to 900 psi
Injection:	0.25 to 2.0 μg in at least 50% mobile phase.

A standard AZT-MP solution was prepared by mixing 20 μl of 1 mg/ml AZT-MP with 380 μl of the mobile phase (0.05 μg/μl), and calibration established by injecting volumes of 5 μl to 40 μl (0.25 to 2.00 μg).

A quantity of 10 μl of the liposome preparation was solubilized with 240 μl of methanol to release all the entrapped AZT-MP, and mixed with an equal volume, 250 μl, of mobile phase for chromatographic analysis. The final solution was clear and had a lipid content of from about 0.5 to 1 mM.

The column was cleaned with 67% methylene chloride/33% methanol, and stored in 100% methanol.

D. Determination of Lipid Phosphorus

A determination of total phosphate was used to determine phospholipid concentration in liposomal preparations which were free of inorganic phosphate ions. Where the liposomal preparation comprises a phosphate buffer, the total liposomal lipids, including phospholipids, were first extracted according to the Bligh-Dyer procedure (Canad. J. Biochem. Physiol., 37:911-917 (1959)).

Phosphate was determined according to the ammonium molybdate method using a commercial phosphorus standard solution of monobasic potassium phosphate, 20 μl inorganic phosphate per ml. (Sigma 661-9, St. Louis, MO).

EXAMPLE 4

Method for Determining Serum-Mediated Leakage

Samples of AZT-MP formulations were mixed with human serum and an adequate volume of PBS to achieve a concentration of 80% human serum and 20 to 100 μM of AZT-MP. Portions of the mixed solutions were incubated at 5°C and 37°C, and samples removed at 2, 24, 48, and 72 hours. Two volumes of methanol and one volume of 2% TFA, both at 5°C, were immediately added to these samples to solubilize the liposomes, precipitate the serum proteins, and stop the conversion of AZT-MP to AZT.

The samples were then centrifuged at 2500 rpm for 15 min at 5°C, and the supernatant withdrawn and analyzed by HPLC on a Vydac C18 reverse phase column, as in Example 3 , except that the gradient was run from 0 to 10% acetonitrile in 20 min.

A solution of free AZT-MP was run in parallel as a control.

On the chromatograph, the AZT-MP peak indicates the drug which remains entrapped, and the AZT peak represents drug which has leaked out and been enzymatically converted to AZT by serum alkaline phosphatase. Control experiments with free drug showed that the phosphatase activity in serum was sufficient to hydrolyze all of the drug rapidly at 37°C. The results of these studies are presented in Figures 10 through 12.

Many other objects, features, and advantages of the present invention will be apparent to those of skill in the art.

Although the invention has been described in the context of certain preferred embodiments, it will be understood that the invention is intended only to be limited by the lawful scope of the claims that follow, and equivalents thereof.

WHAT IS CLAIMED IS:

1. A formulation for preparing liposomes, comprising:

a lipid phase comprising at least one neutral phospholipid species and at least one negatively charged phospholipid species, wherein said negatively charged phospholipids are at least 1 mole percent of total lipids in said lipid phase, and said phospholipids have acyl groups, at least some of which are unsaturated;

an aqueous phase comprising a buffered hydration solution having a pH of from about 4.0 to 8.0; and

a nucleotide species in said buffered solution.

2. A liposomal preparation, comprising:

liposomes having a lamellar structure, said structure comprising at least one neutral phospholipid species and at least one negatively charged phospholipid species, wherein said negatively charged phospholipids are at least 1 mole percent of total lipids in said liposomes, said phospholipids having acyl groups, at least some of which are unsaturated;

a nucleotide species substantially entrapped within said liposomes; and

an aqueous phase comprising a physiologically acceptable buffered solution having a pH between about 4.0 and 8.0.

3. A formulation of Claim 1 or a preparation of Claim 2, wherein said negatively charged phospholipids are at least 5 mole percent of said total lipids.

4. A formulation of Claim 1 or a preparation of Claim 2, wherein said buffered solution has a pH between about 4.0 and 6.0.

5. A formulation of Claim 1 or a praparation of Claim 2, wherein said buffered solution has a pH between about 6.0 and 8.0.

6. A formulation of Claim 1 or a preparation of Claim

2, wherein the nucleotide concentration in said buffered solution is between about 20 mM to 140 mM.

7. The formulation of Claim 1, wherein the nucleotide concentration in the hydration buffer is about 50 mM.

8. A formulation of Claim 1 or a preparation of Claim 2, wherein said nucleotide analogue is AZT monophosphate.

9. A formulation of Claim 1 or a preparation of Claim 2, wherein the lipid phase further comprises cholesterol.

10. A formulation of Claim 1 or a preparation of Claim 2, wherein said negatively charged phospholipid is from about 2 to 4 mole percent of total lipids in said lipid phase, said cholesterol is from about 25 to 35 mole percent of total lipids in said lipid phase, said buffered solution has a pH of from about 7.0 to 8.0; and said nucleotide species is present in said buffered solution at a concentration of from about 20 mM to 90 mM.

11. A formulation of Claim 1 or a preparation of Claim 2 wherein said negatively charged phospholipid is from about 8 to 12 mole percent of total lipids in said lipid phase, said cholesterol is from about 25 to 35 mole percent of total lipids in said lipid phase, said buffered solution has a pH of from about 4.5 to 5.5 and said nucleotide species is present in said buffered solution at a concentration of from about 20 mM to 90 mM.

12. A formulation of Claim 1 or a preparation of Claim 2 wherein the concentration of total lipid with respect to said formulation is between about 90 mM and 300 mM.

13. A formulation of Claim 1 or a preparation of Claim 2 wherein said negatively charged phospholipids are phosphatidyl glycerols.

14. A formulation of Claim 1 or a preparation of Claim 2 wherein said unsaturated acyl groups are oleoyl groups.

15. A formulation of Claim 1 or a preparation of Claim 2 wherein at least one of said unsaturated acyl groups is an oleoyl group and at least one other is a palmitoyl group.

16. The formulation of Claim 1 wherein the osmolarity

of the hydration buffer is greater than about 200 mOs.

17. The formulation of Claim 16, wherein the osmolarity is due, at least in part, to either sodium chloride or a neutral sugar.

18. A process for preparing liposomes, comprising:

providing a lipid phase comprising at least one neutral phospholipid species and at least one negatively charged phospholipid species, wherein the negatively charged phospholipids are at least about 1 mole percent of total lipids in said lipid phase, and said phospholipids have acyl groups, at least some of which are unsaturated;

providing a hydration buffer having a pH between about 4.0 and 8.0 and having a nucleotide species solubilized therein;

contacting said lipid phase with said hydration buffer, whereby primary liposomes are formed; and

adjusting the size of said primary liposomes by extrusion through a filter of uniform pore size.

19. A process of Claim 18, further comprising the step of freezing and then thawing the liposome preparation prior to the extrusion step.

20. A process of Claim 19, wherein the steps of freezing and thawing followed by extrusion are repeated at least once.

21. A process of Claim 20 further comprising repeating the extrusion step at least once.

22. A process of Claim 18, wherein said filter has a uniform pore size of about 0.200 microns.

23. Liposomes prepared according to the process of any one of Claims 18 to 22.

24. Liposomes according to Claim 23 wherein said liposomes are substantially the same size.

25. Liposomes according to Claim 24, wherein a substantial number of said liposomes are approximately 200 microns in diameter.

26. Liposomes according to Claim 23 wherein said liposomes are substantially unilamellar.

27. Liposomes according to Claim 23 wherein said liposomes have substantial stability against leakage of entrapped nucleotide, retaining over 90% of said nucleotide within the liposomes for a period of at least 6 months when stored at temperatures below about 15°C.

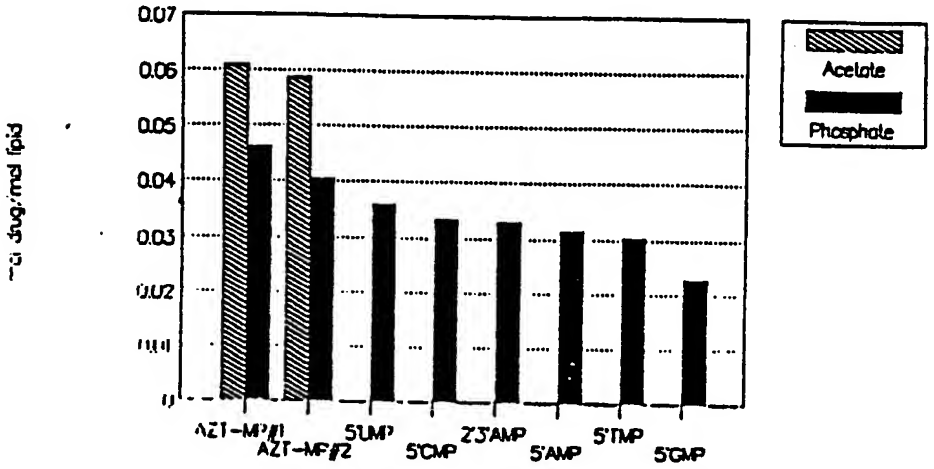
28. Liposomes according to Claim 23 wherein said liposomes have substantial stability in serum against leakage of entrapped nucleotide, retaining over 75% of said nucleotide within the liposomes for a period of at least about 24 hours when exposed to a solution containing approximately 80% serum at about 37°C.

29. Liposomes according to Claim 23 wherein said liposomes have substantial stability in serum against leakage of entrapped nucleotide, retaining more than about 50% of said nucleotide within the liposomes for a period of at least 24 hours when exposed to a solution containing approximately 80% serum at about 37°C.

WO 90/14074 PCT/US90/02858

FIGURE 1

Encapsulation of 50mM AZT-MP and nucleo-side monophosphates into .2um liposomes



Encapsulation of 50mM AZT-MP and nucleo-side monophosphates into .2um liposomes

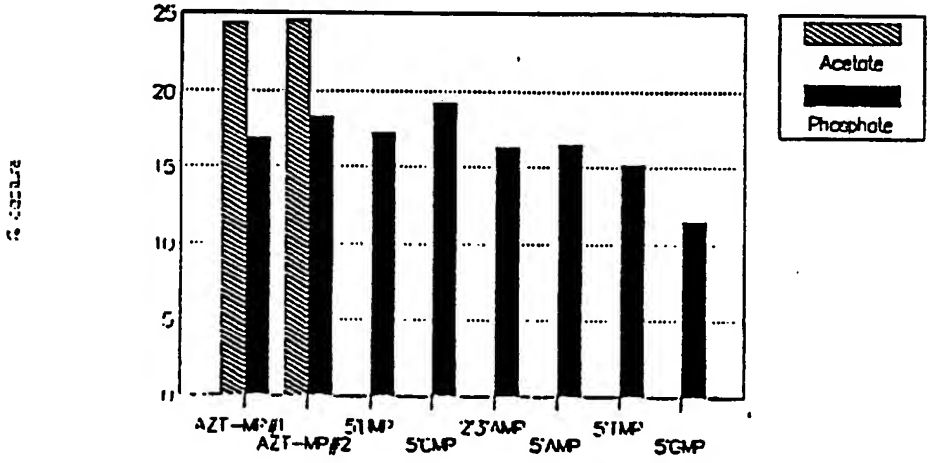


FIGURE 2

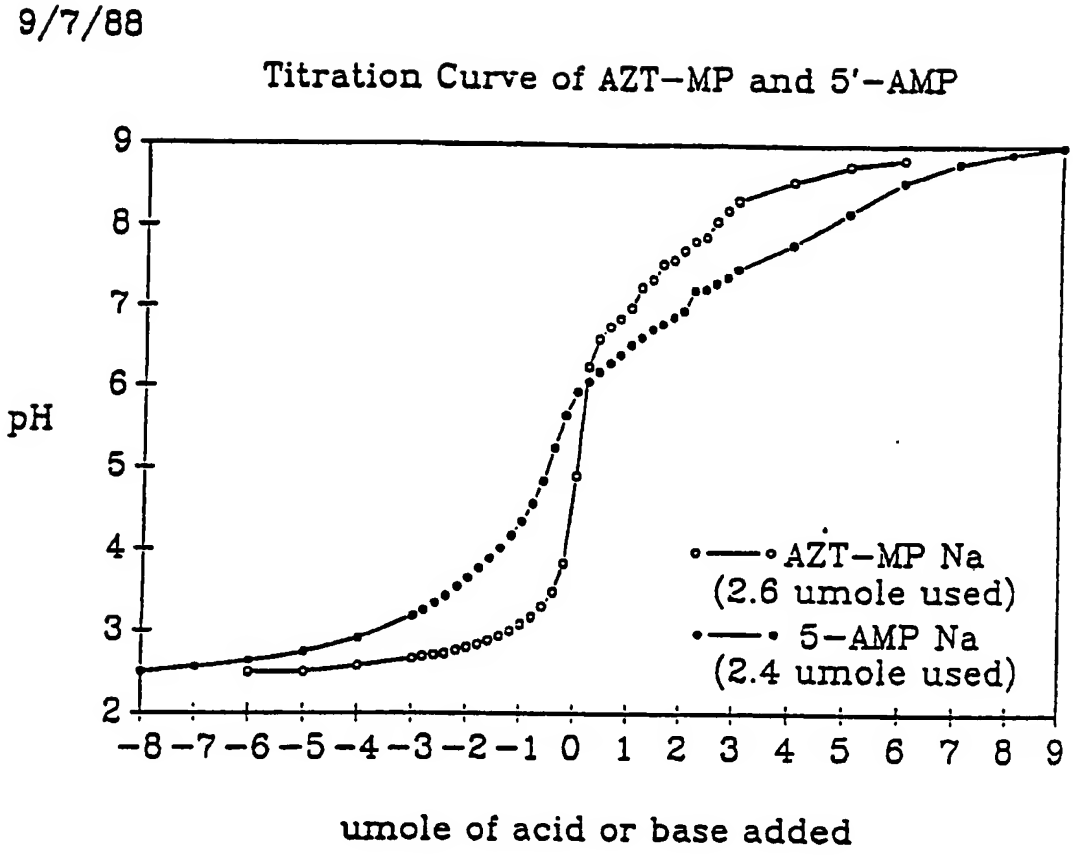

9/7/88
Titration Curve of AZT-MP and 5'-AMP
pH
9
8
7
6
5
4
3
2
-8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9
AZT-MP Na
(2.6 umole used)
5-AMP Na
(2.4 umole used)
umole of acid or base added

FIGURE 3

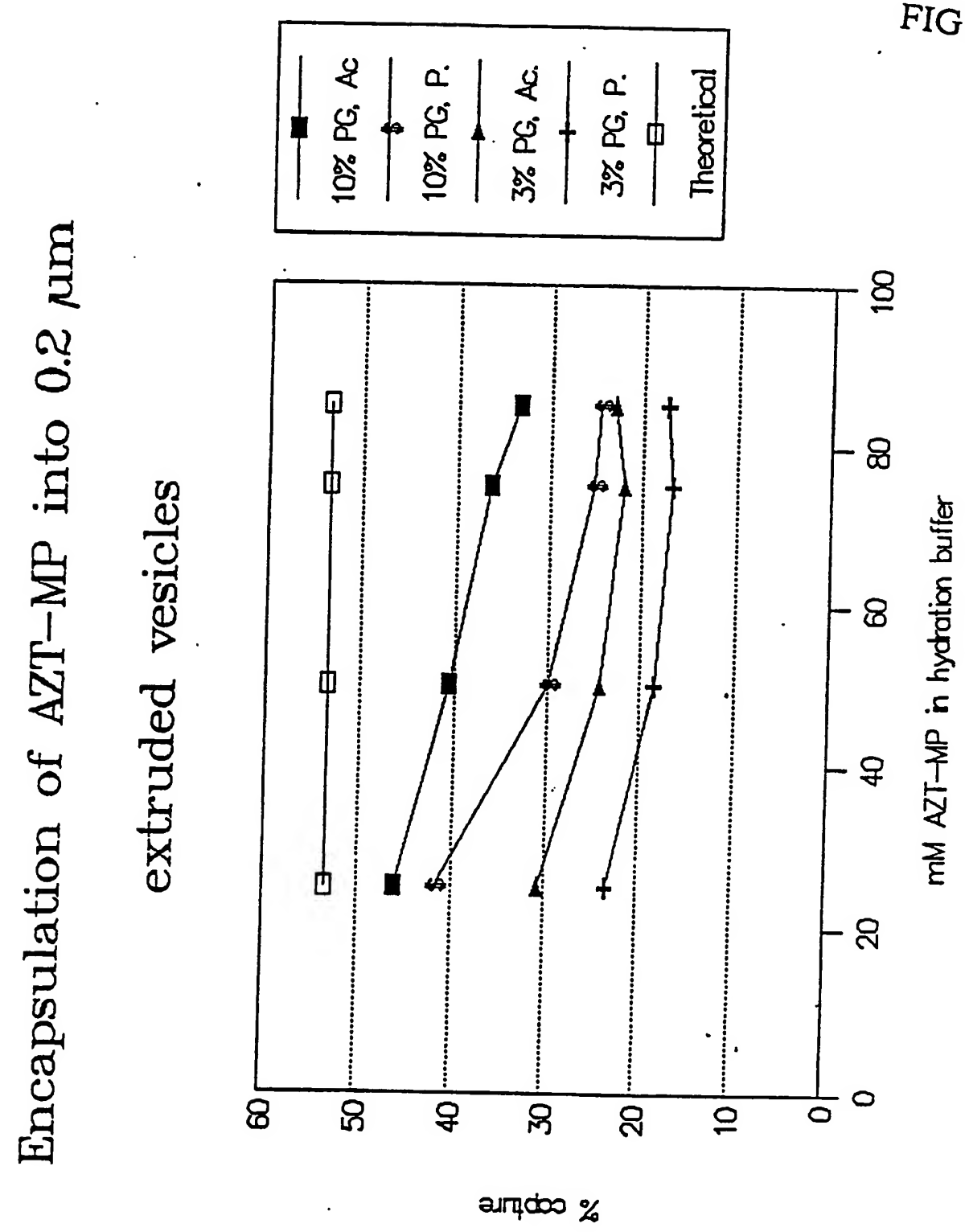
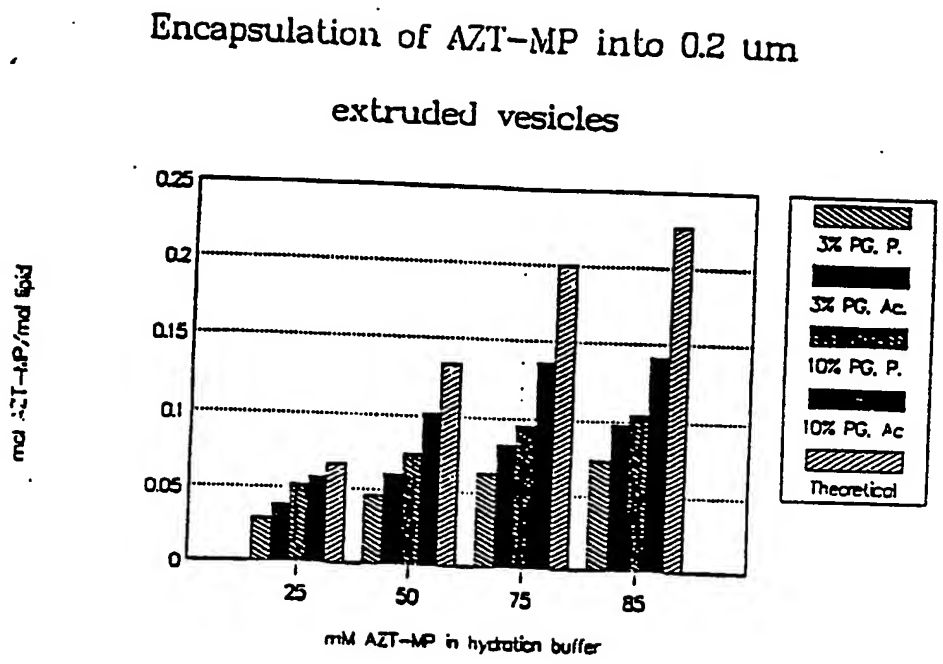

Encapsulation of AZT-MP into 0.2 μm
extruded vesicles
10% PG, Ac
10% PG, P.
3% PG, Ac.
3% PG, P.
Theoretical
% capture
0
10
20
30
40
50
60
0
20
40
60
80
100
mM AZT-MP in hydration buffer

FIGURE 4


Encapsulation of AZT-MP into 0.2 um extruded vesicles
mol AZT-MP/mol lipid
0.25
0.2
0.15
0.1
0.05
0
25
50
75
85
mM AZT-MP in hydration buffer
3% PG, P.
3% PG, Ac.
10% PG, P.
10% PG, Ac
Theoretical

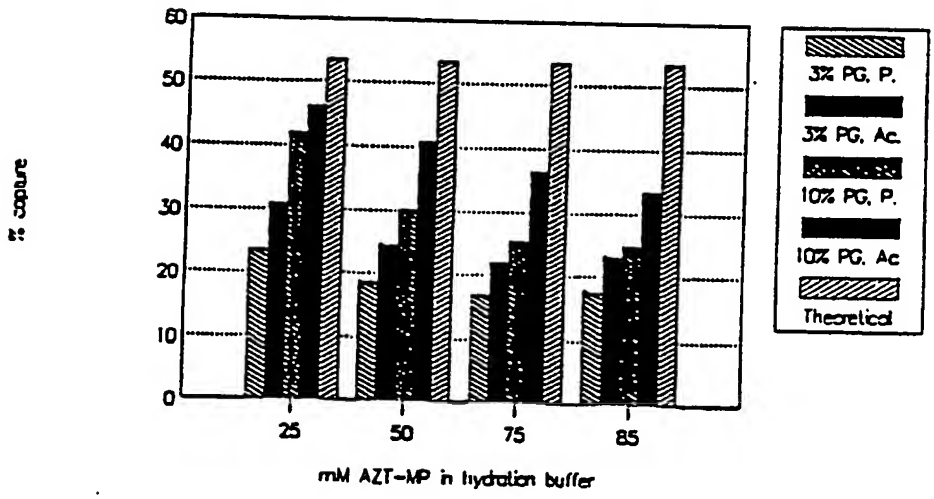
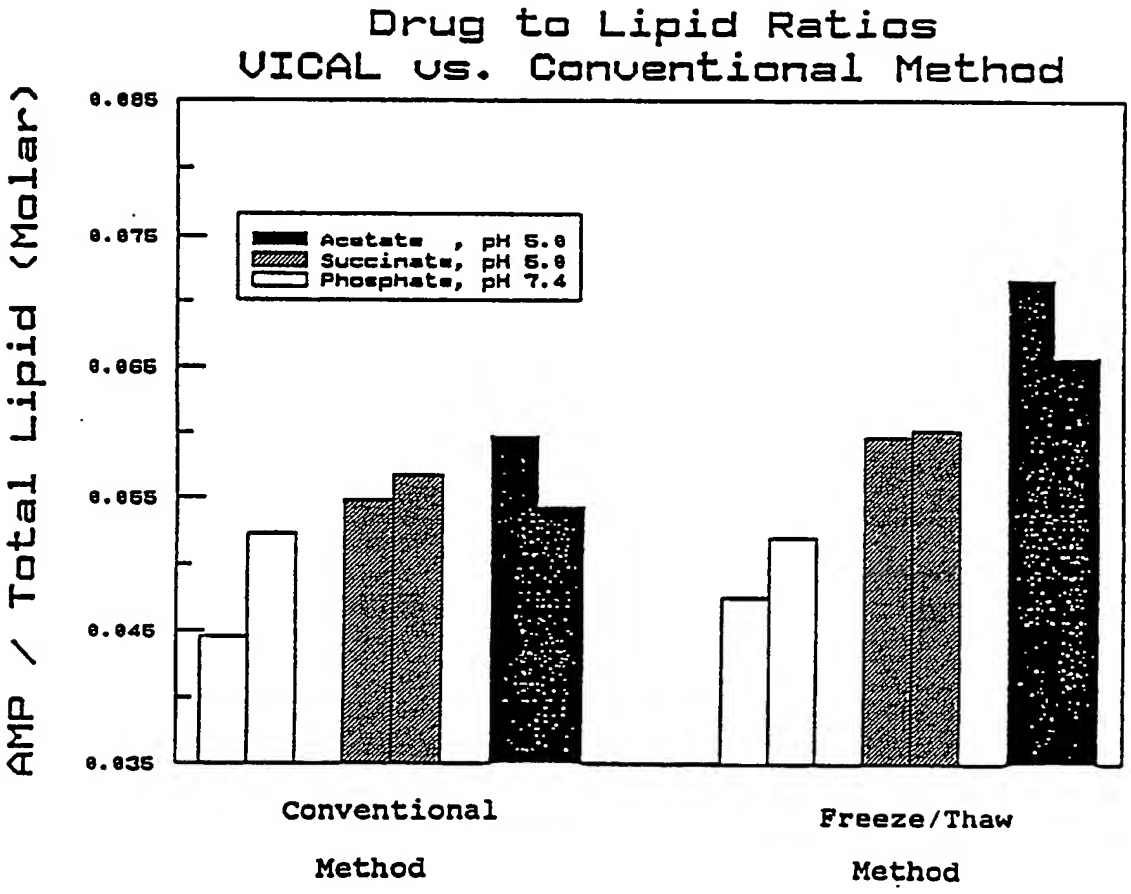

Encapsulation of AZT-MP into 0.2 um extruded vesicles
% capture
60
50
40
30
20
10
0
25
50
75
85
mM AZT-MP in hydration buffer
3% PG, P.
3% PG, Ac.
10% PG, P.
10% PG, Ac
Theoretical

FIGURE 5



The first bar in each pair represents NaCl
The second bar is for Sorbitol

Percent Encapsulation FIGURE 6

pH 5.0 vs 7.4

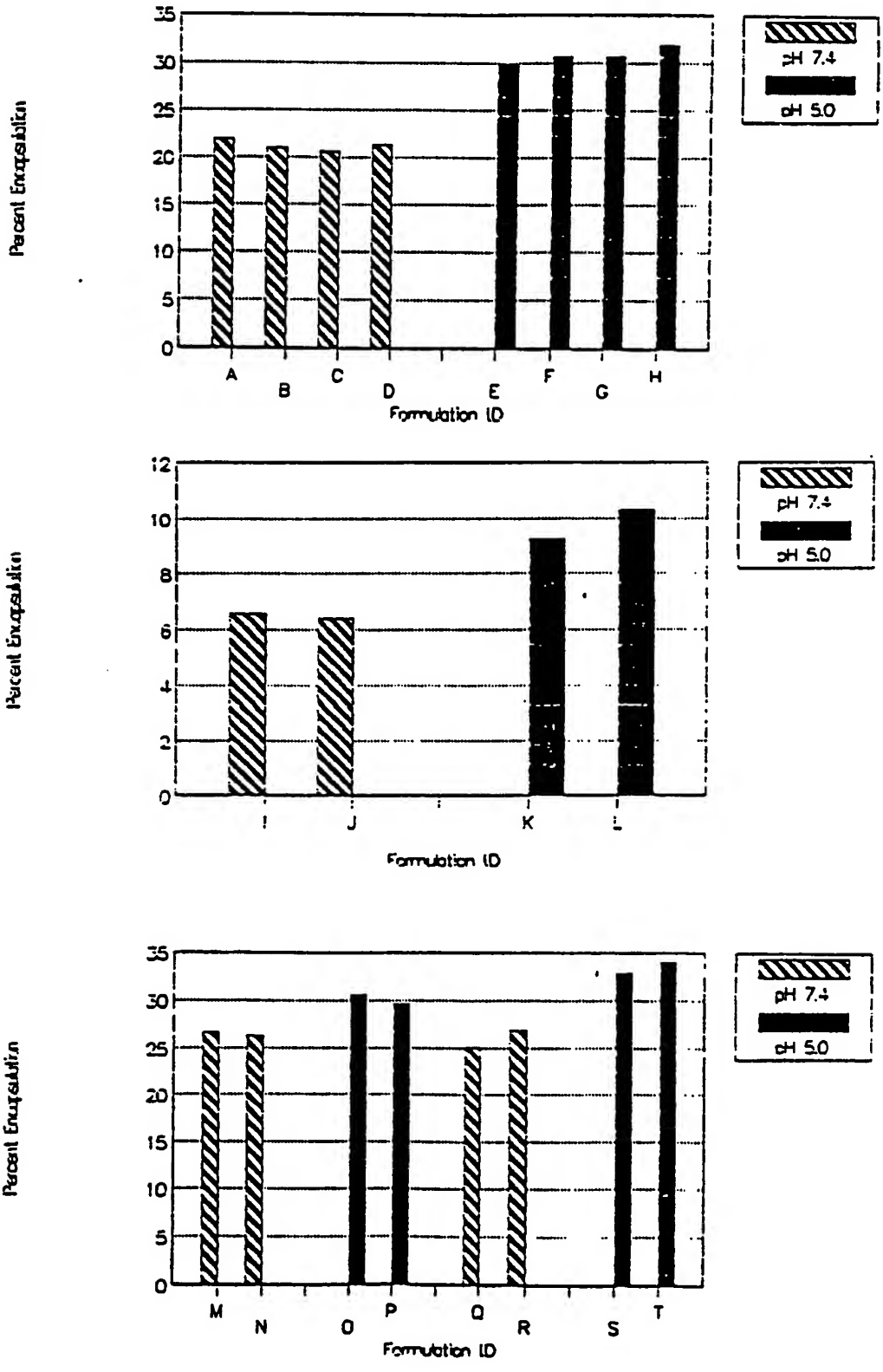
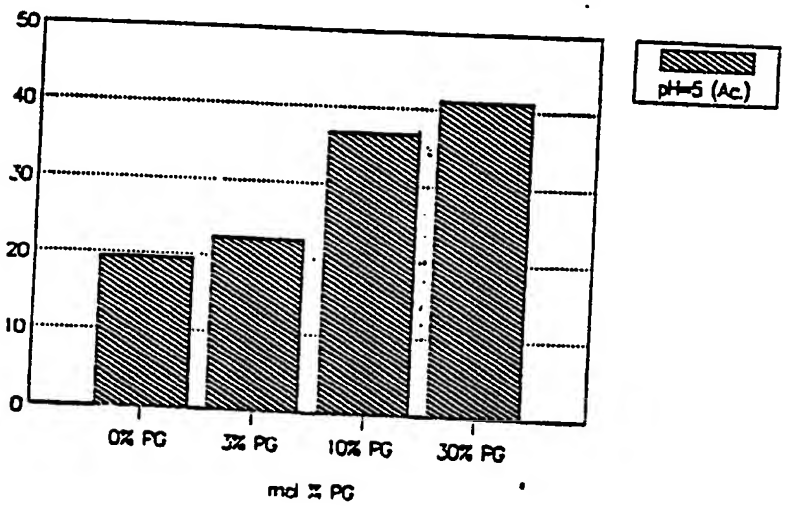


FIGURE 7

Encapsulation of 50mM AZT-MP into 0.2um negatively charged extruded vesicles



Encapsulation of 50mM AZT-MP into 0.2um extruded vesicles

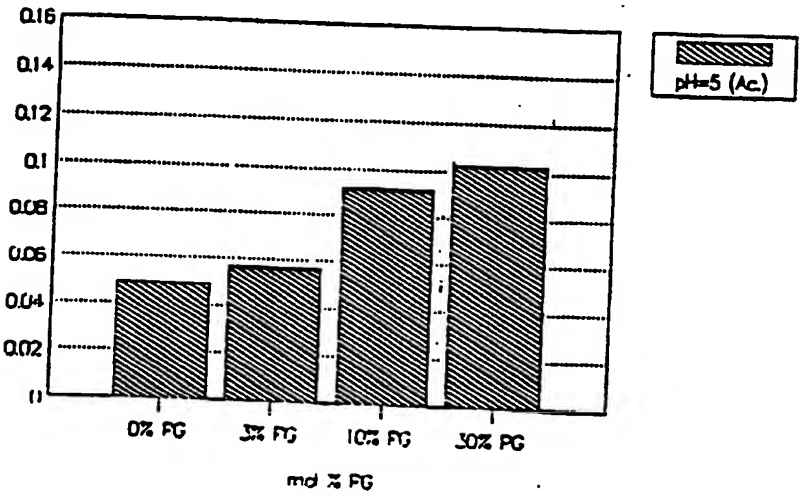


FIGURE 8

% Free AZT-MP at 5 Degrees C

6 Months Stability

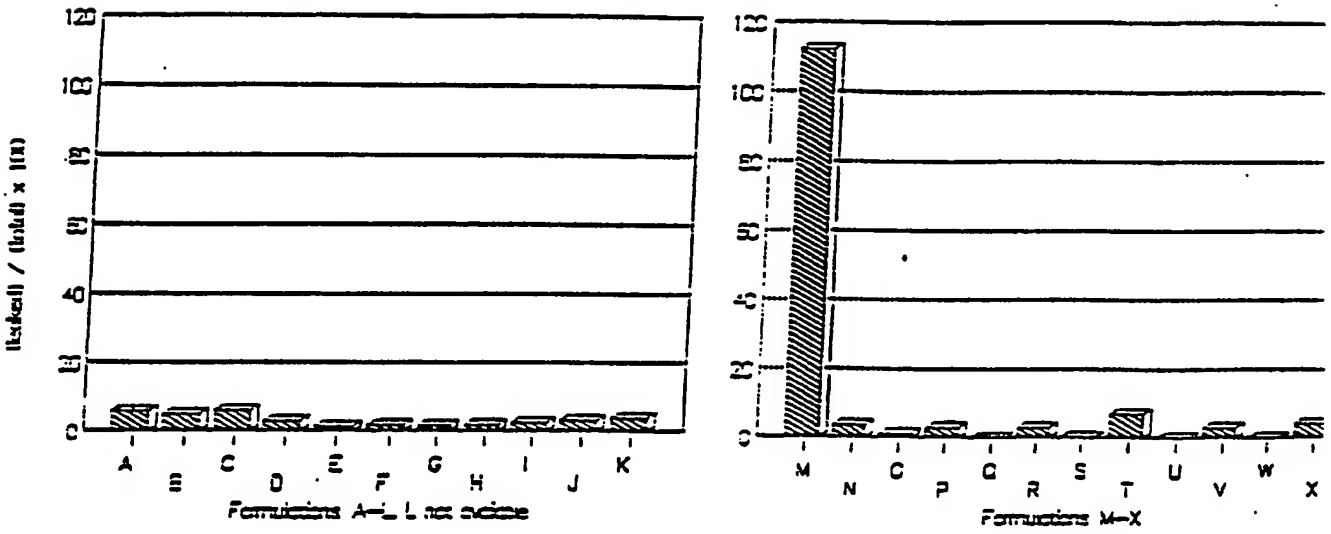
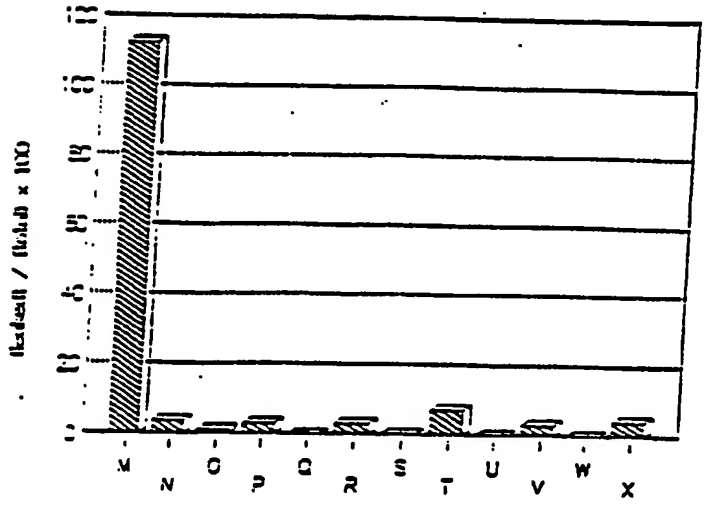


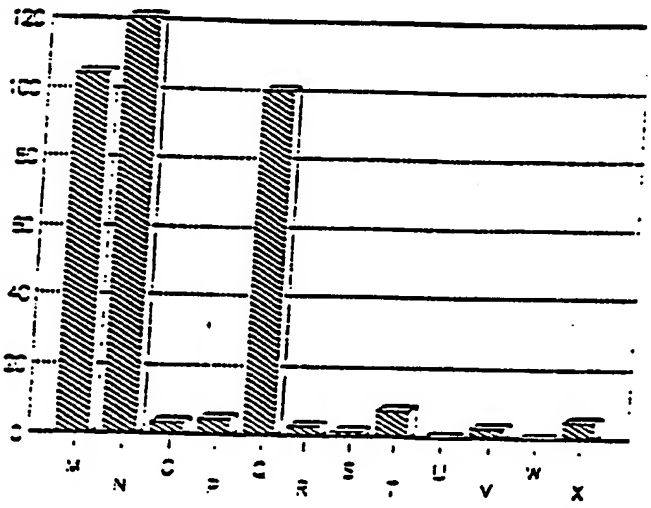
FIGURE 9

% Free AZT-MP - 6 Months Stability
Formulations M-X

5 Degrees C



15 Degrees C



25 Degrees C

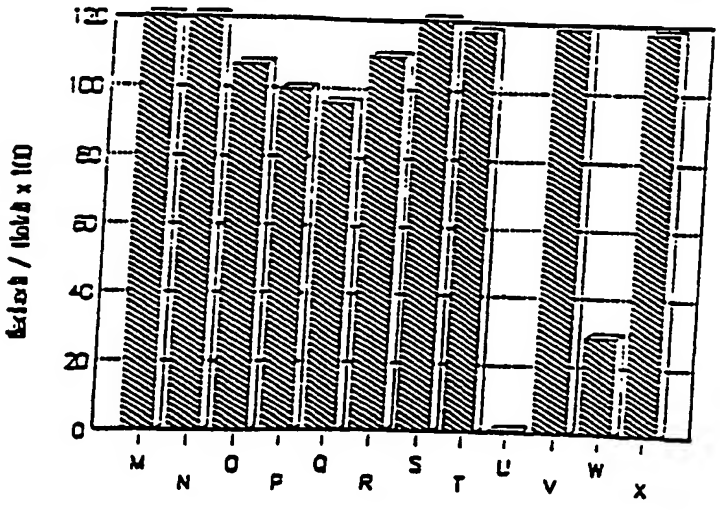
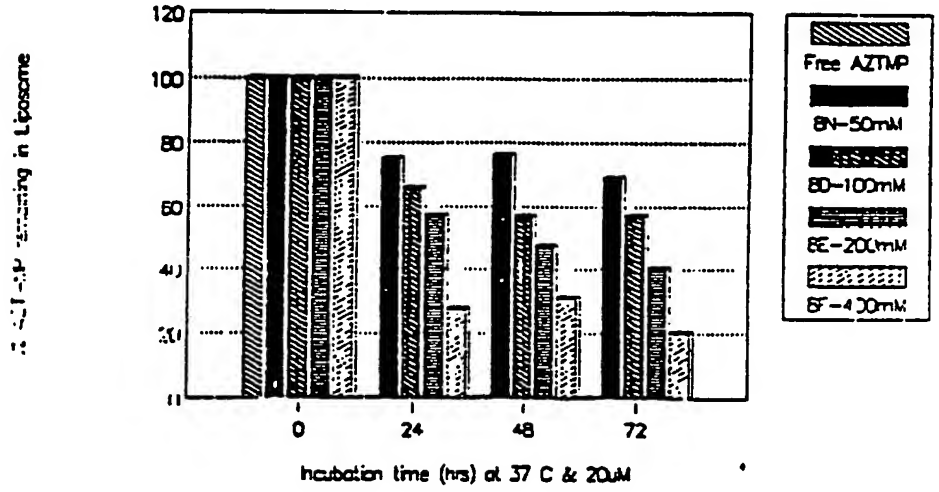


FIGURE 10

Effect of Drug Concentration on EPC40 Liposome Stability in 80% Human Serum



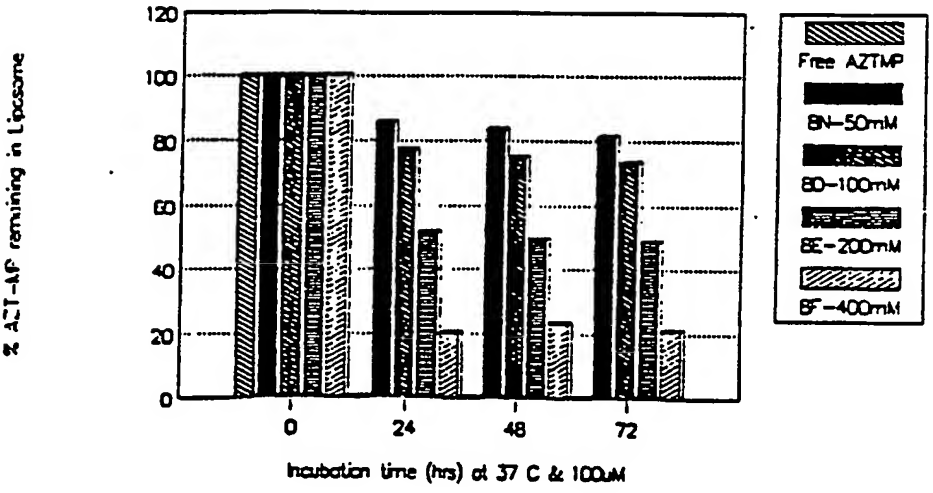
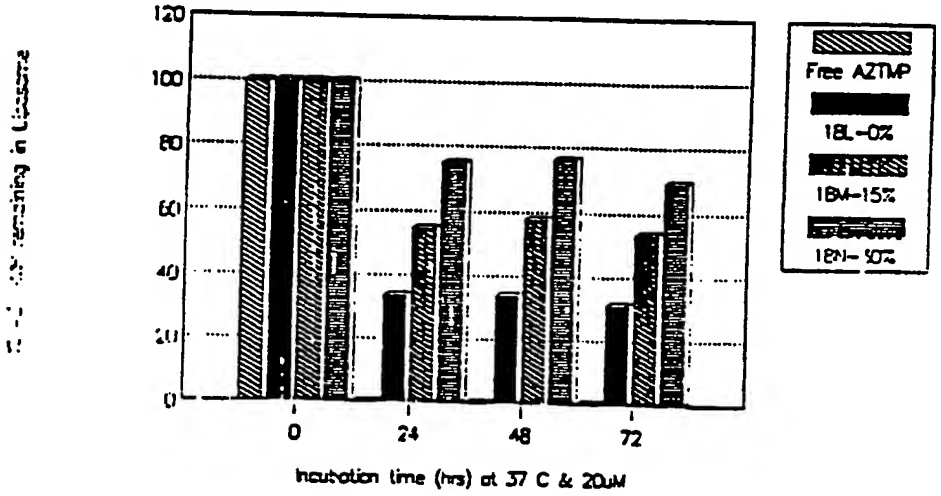


FIGURE 11

Effect of mole % Cholesterol on EPC40 Liposome Stability in 80% Human Serum



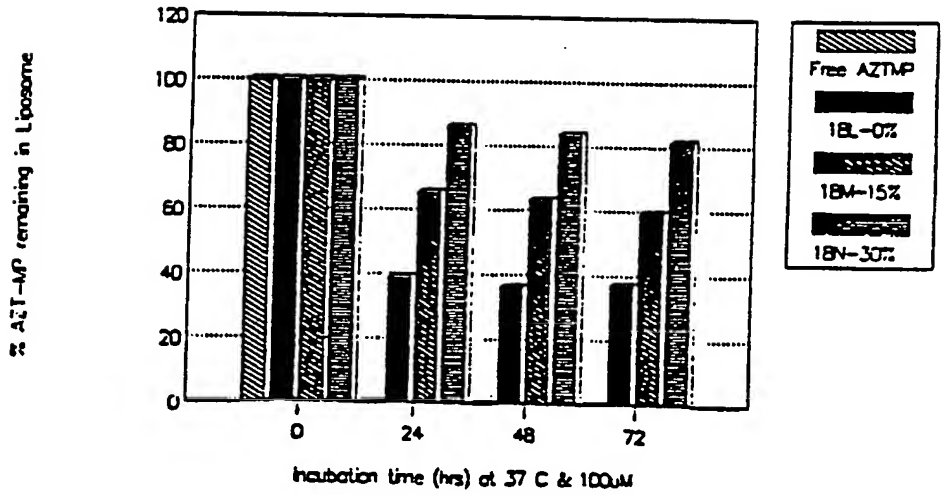
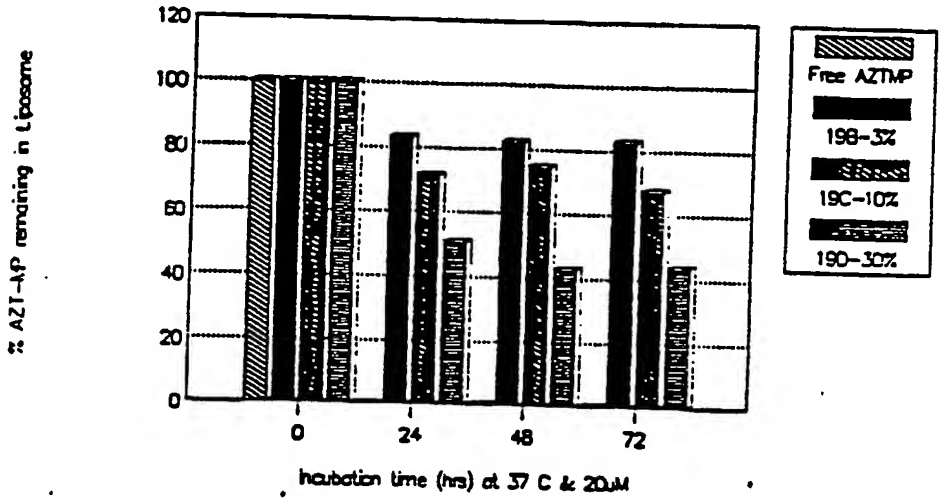
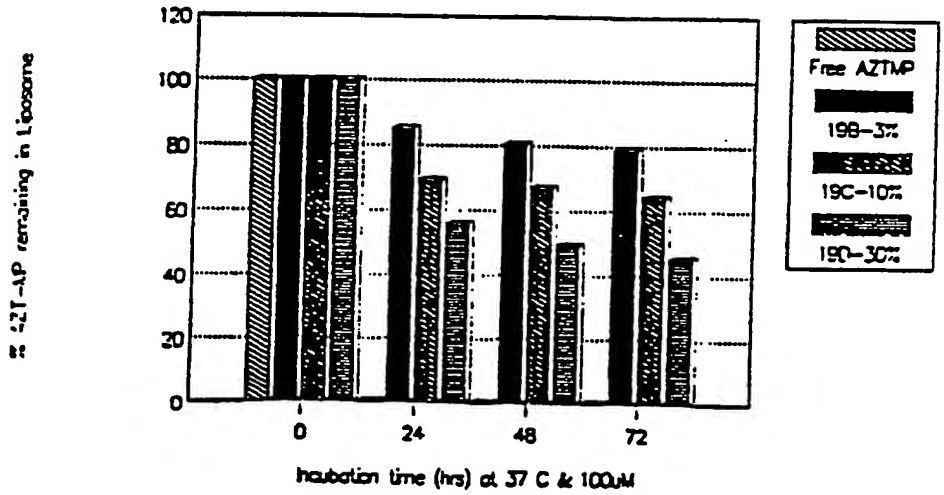


FIGURE 12

Effect of mole % DOPG on POPC Liposome Stability in 80% Human Serum





INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/02858

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 9/127, 9/133; B01J 13/02

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	424/450; 428/402.2; 264/4.1, 4.6

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [5]

STN Messenger Text Search, File CA, File Biosis Dialog Text Search, File wpi, wpil

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	WO, A, 89/02733 (HOSTETLER et al.) 06 April 1989, see the entire document.	1-17,23-29
A,P	EP, A, 350287 (HOSTETLER et al.) 10 January 1990, see the entire document.	1-17,23-29
Y	US, A, 4,797,285 (BARENHOLZ ET AL.) 10 January 1989, see column 7, line 10 bridging column 9, line 10.	1-17,23-29
Y	US, A, 4,879,277 (MITSUYA ET AL.) 07 November 1989, see column 2, lines 8-32; column 3, lines 46-53; and claims 1-5.	1-17,23-29
Y	US, A, 4,564,599 (JANOFF ET AL.) 14 January 1986, see column 4, lines 7-46; and column 7, lines 60-68.	1-17,23-29
A	US, A, 4,737,323 (MARTIN et al.) 12 April 1988, see the entire document.	18-22
A	US, A, 4,752,425 (MARTIN et al.) 21 June 1988, see the entire document.	18-22

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
09 JULY 1990	15 OCT 1990

International Searching Authority [1]	Signature of Authorized Officer [11]
ISA/US	For JOHN M. COVERT Minh Ha Nguyen-Ngoc INTERNATIONAL DIVISION

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	J. Molecular Biology, volume 13, published 1965, A.D. Bangham et al., "Diffusion of Univalent Ions across the Lamellae of Swollen Phospholipids", pages 238-252, (Eng).	18-22
A	Biochim Biophys Acta, volume 817, published 1985, L.D. Mayer et al., "Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles", pages 193-196, (Eng).	18-22
Y	Biochim Biophys Acta, volume 858, published 1986, L.D. Mayer et al., "Vesicles of Variable sizes produced by a rapid extrusion procedure", pages 161-168, (Eng).	18-22

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . , because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.